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Effective delivery of recombinant proteins to rod photoreceptors via lipid nanovesicles

Sabrina Asteriti [a, 1], Giuditta Dal Cortivo [b, 1], Valeria Pontelli [c], Lorenzo Cangiano [a], Mario Buffelli [c, d, *, 2], Daniele Dell'Orco [b, d, *, 2]

[a] Dept. of Translational Research, University of Pisa, Pisa, Italy
[b] Dept. of Life Sciences and Reproduction, University of Verona, Strada Le Grazie 8, Verona, Italy
[c] Dept. of Neurological and Movement Sciences, University of Verona, Strada Le Grazie 8, Verona, Italy
[d] Center for Biomedical Computing, University of Verona, Strada le Grazie 8, 37134 Verona, Italy

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ABSTRACT

The potential of liposomes to deliver functional proteins in retinal photoreceptors and modulate their physiological response was investigated by two experimental approaches. First, we treated isolated mouse retinas with liposomes encapsulating either recoverin, an important endogenous protein operating in visual phototransduction, or antibodies against recoverin. We then intravitrally injected *in vivo* liposomes encapsulating either rhodamin B or recoverin and we investigated the distribution in retina sections by confocal microscopy. The content of liposomes was found to be released in higher amount in the photoreceptor layer than in the other regions of the retina and the functional effects of the release were in line with the current model of phototransduction. Our study sets the basis for quantitative investigations aimed at assessing the potential of intraocular protein delivery via biocompatible nanovesicles, with promising implications for the treatment of retinal diseases affecting the photoreceptor layer.

1. Introduction

Blindness remains one of the most dreaded diseases due to its dramatic impact on the quality of life of patients. Many early-onset forms of retinal disease are caused by inherited mutations in key proteins of the phototransduction cascade, which lead to deregulation of calcium levels and, sooner or later, to photoreceptor degeneration. In recent years a large number of studies have focused on the delivery of small therapeutic compounds, or even entire genes, to the retina (see for instance [1]). An alternative to gene therapy that would allow a better control of the outcome in terms of desired functional protein levels in photoreceptors, would be the direct delivery of intact and functional proteins. A 'protein therapy' approach could exploit the many advantages of the recombinant protein technology, allowing to compensate for the lack of native proteins and even outcompete the dysfunctional ones. One major drawback of protein therapy is that delivery can be rather challenging. Delivery of proteins via the blood stream, for instance, would lead to protein degradation, and successful attempts have been limited to compartmentalized areas of selected tissues like the brain or the heart. Proteins, however, could be encapsulated in lipid nanovesicles (liposomes) and delivered directly to the eye. Lipid-based nano-devices, including liposomes, have been shown to reduce the toxicity and increase the residence time of several active molecules in the eye, and can effectively protect their content from degradation [2–4]. Successful reports have shown the potential of liposomes for improving the treatment of a variety of eye diseases [5,6].

Here we explored the possibility of using 130 nm liposomes as carriers of high amounts of recombinant proteins in the posterior segment of the eye, in order to exert a specific physiological function. We used a lipid composition that ensures full biocompatibility while guaranteeing stability of the suspension. Liposomes were

* Corresponding authors. Center for Biomedical Computing, University of Verona, Strada le Grazie 8, 37134 Verona, Italy.
E-mail addresses: mario.buffelli@univr.it (M. Buffelli), daniele.dellorco@univr.it (D. Dell'Orco).
[1] Co-first author.
[2] These authors contributed equally to this work.

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encapsulated with recombinant proteins affecting the phototransduction cascade. By *in vitro* acute treatment of mouse retinas and following electrophysiological recordings, we demonstrated that the release of the nanovesicle content within rod photoreceptors alters phototransduction in a way that is quantitatively in line with the current most comprehensive model of phototransduction kinetics. Moreover, we found that one intravitreal injection *in vivo* is sufficient to accumulate high amount of recombinant proteins in the photoreceptor layer.

2. Materials and methods

2.1. Preparation of recombinant proteins

Recombinant myristoylated recoverin (Rec) was expressed and purified as described in a previous work [7]. Details on the specific protocols are reported in the Supplementary Materials.

2.2. Preparation and characterization of liposomes with different lipid content

Lipid films for the preparation of lipid vesicles following hydration with physiological buffers were obtained by mixing suitable amounts of L-α-phosphatidylcholine from egg (PC, Sigma Aldrich) and cholesterol (Ch, Sigma Aldrich) dissolved in chloroform in order to obtain 8:1 w/w (PC:Ch 8:1) and PC:Ch 60:40 mixtures. Samples were aliquoted to get 2 mg lipid films per aliquot and dried down by vacuum in a speed-vac concentrator. Dried lipid films were frozen and stored at −80 °C until use. Liposomes incapsulating rhodamine B (RhB-Lip), Rec (Rec-Lip) and antibodies against recoverin (anti-Rec-Lip) were prepared and characterized by dynamic light scattering (DLS) as detailed in the Supplementary Materials.

2.3. Electrophysiology

Dark adapted mice were anesthesized by i.p. injection of urethane 20% W/V in 0.9% saline. Both retinas were rapidly extracted through corneal incisions into cold bicarbonate-buffered Ames' medium, the vitreous removed with forceps and each laid vitreal side down in a Petri dish containing 2 ml of Ames' supplemented with 10% specific liposome solution. The experimenter was blind as to the contents of the two dishes, which were prepared and labeled anonymously by another person. Both dishes were placed uncovered in the same chamber, which was continuously aerated with a 95% O_2/5% CO_2 mixture, and incubated at 37 °C for 2 h. Thereafter, the retinas were rinsed with Ames', laid down next to each other on filter paper and sliced as previously described [8]. Slices were transferred to the recording chamber and superfused with bicarbonate-buffered Ames' medium at ~24 °C. Recordings were targeted to slice areas displaying intact rod outer segments using a blind 'perforated loose seal' technique that does not alter rod response kinetics [9]. Recordings from the two retinas were interleaved, so as to compensate for any time-dependent effects on the properties of rods. Full field flashes of 1–10 ms duration and 520 nm wavelength were delivered as previously described [9]. Data are reported as mean and SEM or 95% confidence interval ($CI_{95\%}$). Statistical significance was assessed with the Mann-Whitney-Wilcoxon (MWW) test, the extra-sum-of-squares F-test (F-test) and the Kruskal–Wallis (KW) test.

2.4. Animals, surgery procedures for intravitreal injection, perfusion procedures, and immunoflourescence

The adult C57BL/6J mice (>P30) were anesthetized with 2,2,2-tribromoethanol (Sigma–Aldrich, St Louis, MO, USA); ip dose of 0.4 g/kg body weight and the eyes of control and experimental animals were injected intravitreally with either empty liposomes (2 mg/ml) or RhB-Lip (2 mg/ml) or Rec-Lip (1 mg/ml): each injection consisted of 1 μl of liposome stock solution (see above). Injections were performed, as described in Ref. [10], by using a heat-pulled glass capillary connected to a picospritzer (Parker Instrumentation, NH, USA).

At the end of the experiment, mice were sacrificed by terminal anesthesia and transcardially perfused with 4% paraformaldehyde in PBS, pH 7.4. The eyes were removed, postfixed and retina cryosections (10–20 μm thickness) were permeabilized, stained with antibodies and by DAPI (see Supplementary Materials for details). Acquisitions were performed by confocal microscope Leica SP5 and processed as described in the Supplementary Materials.

2.5. Mathematical modeling

A mathematical model developed previously [11] and describing the kinetics of the phototransduction cascade in mouse rods was used to simulate the effects of an excess or the lack of Rec on the photoresponses as a consequence of the delivery of Rec-Lip or anti-Rec-Lip. The conditions for the Rec knock-out case were simulated by setting the initial concentration of Rec to zero, while the excess of Rec was simulated by assuming a 4-fold higher initial concentration of Rec (i.e., 4×10^7 molecules instead of 1×10^7 molecules) with subsequent recalculation of the equilibria involving different states of Rec and rhodopsin kinase (RK) in the dark, similarly to what was previously done in Ref. [12]. *In silico* experiments for both wild type and liposome-modified rods were run as elucidated previously [13], allowing to assess numerical values for T_{sat}, the length of time that a saturating photoresponse remains at greater than 90% of its maximum current suppression, and τ_D, the dominant time constant of recovery from a saturating response, measured as the slope of T_{sat} over logarithmically increasing stimulus intensities (also called Pepperberg plot; see Ref. [14]).

3. Results

To investigate the effectiveness of protein delivery to photoreceptors via liposome encapsulation we used Rec, an endogenous protein regulating the duration of the phototransduction cascade in photoreceptor cells, or its antibody (anti-Rec). This choice allowed us to obtain a quantitative measure of protein incorporation in rods by performing single cell recordings of their flash responses, as well as to detect the protein distribution in the retina with immunofluorescence. We integrated the morphological analysis by also examining the delivery of liposomes loaded with the non-protein fluorescent marker rhodamine B (RhB).

3.1. Liposome suspensions characterization

Although liposomes were prepared following an identical procedure, the quality of the final suspension was found to strongly depend on the encapsulated content. While PC:Ch 60:40 liposomes loaded with RhB were significantly monodisperse with an average diameter of 124 nm as assessed by DLS (Fig. S1B), the same type of liposomes loaded with Rec led to two main populations in the suspension, corresponding to average diameters of 161 nm and 608 nm (Fig. S1A). This could be due to the fact that Rec in its Ca^{2+}-

bound form extrudes its myristoyl group, which serves as an anchor to bind the protein to the membrane in the native intracellular environment [15] and exposes hydrophobic patches. Some Rec molecules that have not been encapsulated in the vesicles may therefore interact with one another while having the myristoyl moiety buried in the bilayer of two or more vesicles, thus bridging between them and leading to the high molecular mass agglomerates observed by DLS. However, the effective weight of high-order aggregates on the photon correlation signal is modest due to the $1/d^6$ scaling of scattered light intensity.

The quality of PC:Ch 8:1 liposomes loaded with anti-Rec was instead rather high, as shown by the high degree of monodispersion (Fig. S1C) that is consistent with a single population of particles with an average hydrodynamic diameter of approximately 130 nm.

3.2. Electrophysiological properties of rods in retinas treated with Rec or anti-Rec liposomes

We tested the efficacy of liposomes as a vehicle of protein delivery to photoreceptors using acutely isolated mouse retinas. In each experiment the two retinas from the same animal were treated in parallel with liposomes loaded either with Rec or anti-Rec. Single mouse rods were recorded in slices (Fig. S2) with a 'perforated loose seal' approach, previously developed by some of us and ideally suited for a quantitative comparison of unperturbed rod response kinetics [8,9]. The recorded signal was a scaled down replica of the full rod photovoltage (in the hundreds of μV range), which was normalized to the maximum response amplitude obtained with saturating flashes. Varying the intracellular levels of Rec in rods is expected to have a marked effect of their speed of recovery from saturation [11,16]. Stimulation protocols consisted of bright saturating flash (1570 photons/μm^2) preceded and followed by identical dim test flashes (16.6 photons/μm^2) to highlight the rod kinetics of recovery from saturation (Fig. 1A). Bright flash response duration (width at 50% of maximal response) was significantly longer in Rec-Lip treated rods (1.76 s, SEM 0.06, n = 10) compared to anti-Rec-Lip treated rods (1.24 s, SEM 0.08, n = 9) ($p < 0.0001$, MWW test). From a subset of rods exposed to bright saturating flashes at multiple intensities we could generate a so-called 'Pepperberg plot', which displays saturation time versus photoisomerizations evoked by the flash (expressed in natural log units) (Fig. 1B). From the slope of linear fit to the data we obtained the dominant time constant of recovery τ_D, which was significantly larger in Rec-Lip treated rods (484 ms, SEM 76, n = 3) compared to anti-Rec-Lip treated ones (307 ms, SEM 31, n = 4) ($p < 0.05$; F-test). Flashes of increasing intensity were also delivered to rods in each group, and their normalized response amplitudes versus flash strength plotted (Fig. 1C). Hill fits to the data (with shared slope factors) were employed to estimate the half saturating flash strength in each of the two rod groups (a measure of their sensitivity). This parameter was significantly lower (implying higher sensitivity) in Rec-Lip treated rods (9.7 photons/μm^2, $CI_{95\%}$ 8.2–11.5, n = 6) compared to anti-Rec-Lip treated rods (15.9 photons/μm^2, $CI_{95\%}$ 13.5–18.9, n = 6) ($p < 0.0001$; F-test).

To exclude that the effects just described were simply due to the differences in liposome surface composition (PC:Ch 60:40 vs. PC:Ch 8:1) and not to their encapsulated content (Rec vs. anti-Rec), we tested empty liposomes. The retinas from the each animal were treated in parallel with the two types of liposomes. A portion of one retina was also treated with the same extracellular solution but devoid of liposomes. Bright flash response duration was not significantly different between 60:40-Lip (1.68 s, SEM 0.06, n = 6), 8:1-Lip (1.73 s, SEM 0.06, n = 8) and Lip-free treated rods (1.97 s, SEM 0.13, n = 6) ($p = 0.12$, KW test). Crucially, comparison of 60:40-Lip and 8:1-Lip treatment showed no evidence of a differential effect ($p = 0.95$, MWW test) (Fig. 2D). Interestingly, a possible effect of empty liposomes on rod photoresponses cannot be ruled out as indicated by comparing 60:40-Lip ($p = 0.06$) and 8:1-Lip ($p = 0.11$) to Lip-free. The dominant time constants of recovery τ_D, obtained from a Pepperberg plot, were not significantly different between the three treatments ($p = 0.34$; F-test), nor when directly comparing 60:40-Lip (n = 5) and 8:1-Lip (n = 5) ($p = 0.30$).

3.3. In vivo retinal biodistribution of liposomes encapsulating RhB or Rec

To evaluate *in vivo* the liposome distribution in the retina, as well as to check whether encapsulation with a protein would affect the delivery, liposomes loaded with either a fluorescent dye (RhB-Lip) or relatively high amounts of recombinant Rec (Rec-Lip) were injected intraocularly in 8 mice. Intravitreal (ivit) injections of 1 μl liposome solution (4 mice with RhB-Lip and 4 mice with Rec-Lip, see Materials and Methods for details) were followed after 24 h by dissection and analysis of the retinas at the confocal microscope, after staining with appropriate antibodies and with DAPI to label the nuclei. As it is clearly visible in representative images (Figs. S3 and S4), there was an increase in fluorescence in the photoreceptor layer in liposome-injected retinas compared to control animals and to animals treated with empty liposomes (Fig. S3). In particular, in mice treated with RhB-Lip, we found that the fluorescent dye was present at a low concentration in all retinal layers, but surprisingly, the concentration was higher in the outer segment layer of photoreceptors than in other regions.

Fig. 2 shows the distribution of Rec in Rec-Lip-treated and control mice after staining with DAPI and antibodies against Rec

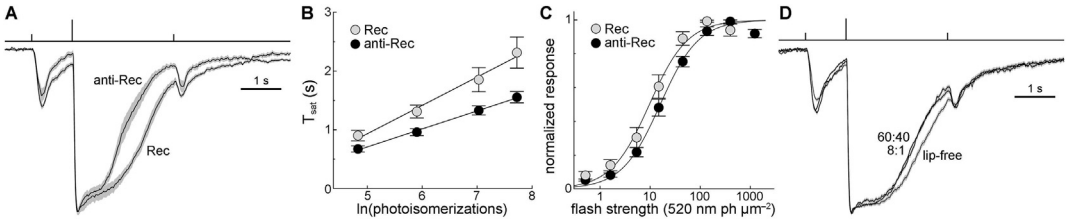

Fig. 1. Rec and anti-Rec loaded liposomes differentially modify rod light sensitivity and kinetics of recovery from saturation. A: Normalized rod photovoltages recorded with a 'perforated loose seal' technique in retinas pre-incubated either with Rec-Lip (n = 10) or anti-Rec-Lip (n = 9). Flash strengths are given in the Results. B: Pepperberg plots for Rec-Lip treated (n = 3), anti-Rec-Lip treated rods (n = 4). Saturation time was taken as the width of the response at half peak amplitude. The dominant recovery time constants of phototransduction are τ_D = 484 ms (SEM 76) for Rec-Lip treated rods and τ_D = 307 ms (SEM 31) for anti-Rec Lip treated rods. C: Average normalized response amplitude as a function of flash strength in rods treated with Rec-Lip (n = 6) and anti-Rec-Lip (n = 6). D: Control normalized rod photovoltages in 60:40-Lip treated (n = 6), 8:1-Lip treated (n = 8) and Lip-free treated rods (n = 6). All panels show mean ± 1 SEM.

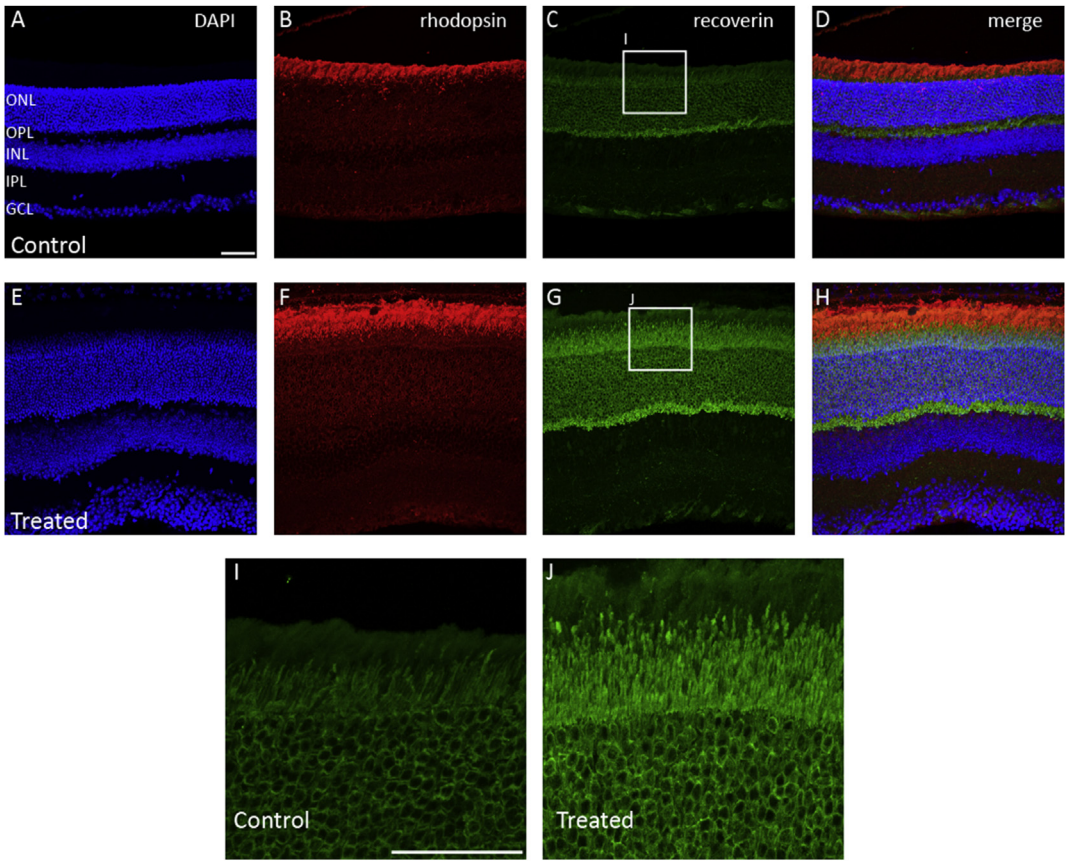

Fig. 2. Biodistribution of Rec in the retina after *in vivo* ivit injection of liposomes containing Rec (Rec-Lip). A–D: Example of untreated (control) mouse retina sections. E–H: Example of mouse retina sections 24 h after a single ivit injection of Rec-Lip. A and E: sections stained with DAPI to label the nuclei; B, F: sections stained with anti-rhodopsin; C, G: sections stained with anti-Rec; I–J: enlargement of two regions in C and G respectively. D, H: merge of blue, red and green images. Control and treated digital photographs were captured using same laser parameters. The distribution of exogenous Rec appeared particularly intense in the inner segment of the photoreceptor layer. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: A-J 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and rhodopsin. In all the treated mice, the pattern of distribution of Rec was the same as that of the endogenous protein, but there was an increase of Rec in the inner segment of photoreceptor layer (Fig. 2G and J) and in the OPL (Fig. 2G). This distribution strongly supports the electrophysiological data.

3.4. Mathematical modeling predicts the effects of protein-loaded liposomes on the photoresponse kinetics

In order to interpret the functional effects observed in the electrophysiological recordings on rod photoreceptors, in the cases of the retinas exposed to Rec-Lip or anti-Rec-Lip, we simulated two extreme cases, whose foundations are elucidated in the Supplementary Materials: (i) all the endogenous Rec molecules are interacting with the anti-Rec carried by the liposomes, thus virtually setting to zero the amount of free intra-rod Rec available for the regulation of the phototransduction cascade; (ii) liposomes carrying extra Rec would increase approximately 4-fold the concentration of intracellular Rec.

Results from the simulations are shown in Fig. 3. A wild type rod was stimulated with flashes ranging from dim intensities (11.8 photons μm^{-2}) up to saturating flashes (1690 photons μm^{-2}; Fig. 3A). When the same flashes were applied to a rod in which Rec had been knocked-out (Fig. 3B), the photoresponses resulted in lower peak amplitudes to dim flashes and significantly reduced T_{sat} for saturating flashes. On the contrary, if a rod with 4-fold higher initial amount of Rec was exposed to the same light stimuli, a significant increase in both peak amplitudes and saturation times could be appreciated (Fig. 3C).

The perturbation of the phototransduction dynamics by different amounts of Rec is best shown by the Pepperberg-plot reported in Fig. 3D, relating the T_{sat} for saturating flashes to the intensity of the stimulating flashes in terms of generated photoisomerizations. While the dominant time constants τ_D predicted by simulations ($\tau_D^{WT} = 242$ ms; $\tau_D^{Rec\_ko} = 201$ ms; $\tau_D^{Rec\_4X} = 291$ ms) are lower compared to those experimentally determined by electrophysiological recordings ($\tau_D = 484$ ms for Rec-Lip treated rods and $\tau_D = 307$ ms for anti-Rec Lip treated rods), the relative time constants are very similar ($\tau_D^{Rec\_ko}/\tau_D^{Rec\_4X} = 0.69$; $\tau_D^{antiRec\_Lip}/\tau_D^{Rec\_Lip} = 0.63$).

4. Discussion

Liposomes are considered increasingly promising in nano-ophthalmology for their high potential in protecting poorly-stable drugs such as peptides and nucleic acids from degradation and limiting the number of intravitreal injections required to achieve therapeutic amounts of drugs [5]. Our study represents the first attempt to use liposomes of nanometric size as carriers of intact proteins to be delivered to retinal photoreceptors, which constitute the target of most vision-threatening diseases.

The suspension of liposomes used in our work was stable for several weeks and presented satisfactory physico-chemical

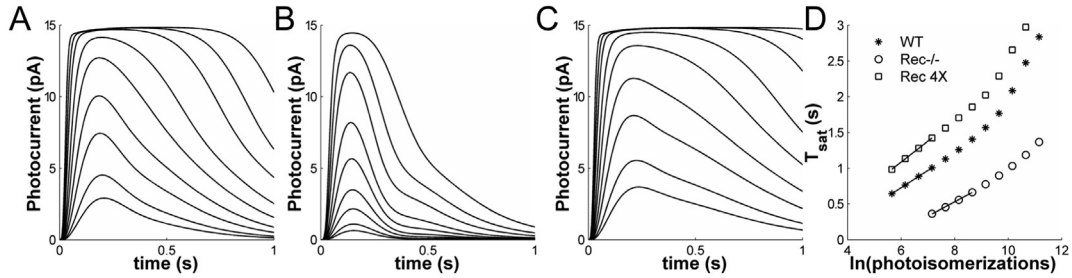

Fig. 3. Effects of variations in the effective concentration of Rec on the photoresponse of a mouse rod according to a recent mathematical model of the phototransduction cascade [11]. A: Simulated photocurrents in a wild type rod stimulated with 20 ms flashes of increasing intensity, from dim to saturating flashes (stimuli intensity: 12, 21, 43, 75, 139, 243, 504, 882, 1690 photons μm^{-2}). B: Simulated photocurrents in a rod in which Rec has been knocked-out, or C: 4-fold over-expressed; light stimuli are the same as for the wild type rod. D: Pepperberg plots. The first four points in each simulated rod experiment were used to determine the dominant time constant (τ_D). Values of τ_D's are the following: $\tau_D^{WT} = 242$ ms; $\tau_D^{Rec\_ko} = 201$ ms; $\tau_D^{Rec\_4X} = 291$ ms.

colloidal properties. The concentrated stocks were generally monodisperse (Fig. S1), with the exception of Rec-Lip, which showed two populations in size distribution, suggesting that the myristoylated protein that has not been encapsulated may act as a bridge between several vesicles. Even in this case though the delivery was successful (Figs. 1 and 2).

The lipid composition of liposomes in our experiments was rather unspecific, being a mixture of selected amounts of PC and Ch to modulate the fluidity of the bilayer. Nonetheless, the *in vivo* distribution of the delivered nanovesicles showed a clear accumulation at the level of the photoreceptors rather than other retina layers (Fig. 2 and Fig. S4). The reason why liposomes that have not been tagged to target specific cells tend to accumulate at the level of the photoreceptors is not completely clear, moreover the mechanism by which vesicles enter the cell remains to be clarified. We speculate that the high spatial density of rods and cones, both characterized by large outer segments, would increase the concentration of membranes of which PC and Ch are natural constituents, therefore facilitating the accumulation and fusion on nanovesicles. Acute electrophysiological recordings performed with the perforated loose seal method (Fig. 1) demonstrate clearly that the modification to the phototransduction cascade, whose molecular machinery is completely intracellular, is due to a fusion of the vesicle within the photoreceptor layer. Interestingly, when the same electrophysiological experiments were performed at a temperature (22 °C) lower than the body temperature (37 °C), no significant difference between treated and untreated retinas could be appreciated (results not shown). Temperature therefore seems to be an important factor in governing vesicle stability and fusion with native membranes. Our data are at odds with a study by Govardovskii et al. [17], in which liposomes loaded with antibodies against the phosphodiesterase affected the phototransduction cascade in frog retinas, without apparently penetrating the cell. In our study in fact, the effect is not reversible, as the retinas were washed after incubation with the liposome suspension. Our data support the hypothesis that the vesicles fuse with the photoreceptor membrane, thereby releasing their content.

The role played by Rec as a regulator of the phototransduction cascade has been known for many years. Rec is a neuronal calcium sensor [18] that, by means of a Ca^{2+}-feedback mechanism, binds and regulates the action of rhodopsin kinase (RK). Previous experiments were performed to specifically evaluate the effect of variations in the amount of Rec on the photoresponse dynamics. Makino et al. [16] recorded photoresponses in transgenic mice not expressing Rec and reported lower peak amplitudes for dim flashes and decreased duration of the photoresponses in saturating conditions. In a different *in vitro* electrophysiological experiment Gray–Keller et al. [19] perfused Rec into functionally intact gecko rods using a whole-cell recording approach and observed the opposite effect, that is a significant increase in both peak amplitude and saturation time. In the present study we qualitatively observed the same behaviors when comparing retinas from the same mouse treated with Rec-Lip or anti-Rec-Lip (Fig. 3). Therefore, liposomes loaded with Rec or its antibody could functionally mimic respectively the case of an intracellular perfusion of extra Rec [19] or sequestration of most of the available endogenous Rec, as it is the case in transegenic mice not expressing Rec [16].

Numerical simulations using a molecular systems biology approach to phototransduction modeling, developed recently by some of us [11–13], enabled us to also run *in silico* experiments alongside the *in vitro* ones. The comparison between simulated data and electrophysiological measurements shows a substantial agreement and poses a boundary to the altered level of intracellular Rec following liposome fusion. In future studies, this modeling approach could be used both to predict the effects of specific liposome loading on the phototransduction cascade and to set parameters for the optimization of the delivery process of the loaded vesicles.

In conclusion, our study provides the proof-of-concept of a novel strategy to deliver full proteins to photoreceptors and modulate phototransduction using biocompatible lipid nanovesicles. Carefully designed recombinant proteins could be used in basic research as an easy to implement probe into the intricacies of the phototransduction cascade. Moreover, the approach presented here opens new scenarios for the development of effective protein therapy approaches to treat retinal diseases.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.088.

Transparency document

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References

[1] L.S. Carvalho, L.H. Vandenberghe, Promising and delivering gene therapies for vision loss, Vis. Res. (2014 Aug 2), http://dx.doi.org/10.1016/j.visres.2014.07.013 pii: S0042-6989(14)00172-2.

[2] J.P. Colletier, B. Chaize, M. Winterhalter, D. Fournier, Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer, BMC Biotechnol. 2 (2002) 9.

[3] S. Meyenburg, H. Lilie, S. Panzner, R. Rudolph, Fibrin encapsulated liposomes as protein delivery system. Studies on the in vitro release behavior, J. Control Release 69 (2000) 159–168.

[4] X. Xu, A. Costa, D.J. Burgess, Protein encapsulation in unilamellar liposomes: high encapsulation efficiency and a novel technique to assess lipid-protein interaction, Pharm. Res. 29 (2012) 1919–1931.

[5] A. Bochot, E. Fattal, Liposomes for intravitreal drug delivery: a state of the art, J. Control Release 161 (2012) 628–634.

[6] A. Rajala, Y. Wang, Y. Zhu, M. Ranjo-Bishop, J.X. Ma, C. Mao, R.V. Rajala, Nanoparticle-assisted targeted delivery of eye-specific genes to eyes significantly improves the vision of blind mice in vivo, Nano Lett. 14 (2014) 5257–5263.

[7] V. Marino, A. Astegno, M. Pedroni, F. Piccinelli, D. Dell'Orco, Nanodevice-induced conformational and functional changes in a prototypical calcium sensor protein, Nanoscale 6 (2014) 412–423.

[8] L. Cangiano, S. Asteriti, L. Cervetto, C. Gargini, The photovoltage of rods and cones in the dark-adapted mouse retina, J. Physiol. 590 (2012) 3841–3855.

[9] S. Asteriti, C. Gargini, L. Cangiano, Mouse rods signal through gap junctions with cones, Elife 3 (2014) e01386.

[10] E. Lorenzetto, M. Ettorre, V. Pontelli, M. Bolomini-Vittori, S. Bolognin, S. Zorzan, C. Laudanna, M. Buffelli, Rac1 selective activation improves retina ganglion cell survival and regeneration, PLoS One 8 (2013) e64350.

[11] B.M. Invergo, D. Dell'Orco, L. Montanucci, K.W. Koch, J. Bertranpetit, A comprehensive model of the phototransduction cascade in mouse rod cells, Mol. Biosyst. 10 (2014) 1481–1489.

[12] B.M. Invergo, L. Montanucci, K.W. Koch, J. Bertranpetit, D. Dell'orco, Exploring the rate-limiting steps in visual phototransduction recovery by bottom-up kinetic modeling, Cell Commun. Signal. 11 (2013) 36.

[13] D. Dell'Orco, H. Schmidt, S. Mariani, F. Fanelli, Network-level analysis of light adaptation in rod cells under normal and altered conditions, Mol. Biosyst. 5 (2009) 1232–1246.

[14] D.R. Pepperberg, M.C. Cornwall, M. Kahlert, K.P. Hofmann, J. Jin, G.J. Jones, H. Ripps, Light-dependent delay in the falling phase of the retinal rod photoresponse, Vis. Neurosci. 8 (1992) 9–18.

[15] J.B. Ames, R. Ishima, T. Tanaka, J.I. Gordon, L. Stryer, M. Ikura, Molecular mechanics of calcium-myristoyl switches, Nature 389 (1997) 198–202.

[16] C.L. Makino, R.L. Dodd, J. Chen, M.E. Burns, A. Roca, M.I. Simon, D.A. Baylor, Recoverin regulates light-dependent phosphodiesterase activity in retinal rods, J. Gen. Physiol. 123 (2004) 729–741.

[17] V.I. Govardovskii, I.L. Dumler, Effect of liposomes containing antibodies to cyclic nucleotide phosphodiesterase on the receptor potential of rods and cones of the frog retina, Neirofiziologiia 17 (1985) 245–250.

[18] R.D. Burgoyne, Neuronal calcium sensor proteins: generating diversity in neuronal Ca^{2+} signalling, Nat. Rev. Neurosci. 8 (2007) 182–193.

[19] M.P. Gray-Keller, A.S. Polans, K. Palczewski, P.B. Detwiler, The effect of recoverin-like calcium-binding proteins on the photoresponse of retinal rods, Neuron 10 (1993) 523–531.